Comparison of Antioxidant Activity of Ethanolic Extracts of Propolis Obtained by Different Extraction Methods

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The antioxidant activities of the ethanolic extracts of propolis obtained by different extraction methods (high hydrostatic pressure extraction, leaching at room temperature and heat reflux extraction) were investigated in relationship to their total polyphenol and flavonoid contents by two different assays, namely, the β-carotene bleaching and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay systems. The results showed that the ethanolic extracts of propolis obtained by high hydrostatic pressure extraction and leaching at room temperature had relatively strong antioxidant activities, which may be correlated with the total polyphenol and flavonoid contents. Antioxidant activities of ethanolic extracts of propolis obtained by high hydrostatic pressure extraction were the same as those of ethanolic extracts of propolis obtained by leaching at room temperature. Leaching at room temperature usually needs a few days, and can take even more than 7 d, while high hydrostatic pressure extraction needs only 1 min. These findings further illustrate that the high hydrostatic pressure extraction has a bright prospect for extracting flavonoids from propolis.

Les activités antioxydantes des extraits d'éthanol obtenus par différentes méthodes d'extraction (extraction par pression hydrostatique élevée, lessivage à température ambiante et extraction par reflux de chaleur) ont été étudiées en relation avec leur teneur totale en polyphénol et flavonoïde par deux essais différents, à savoir les méthodes d'essai par blanchiment du ß-carotène et par entraînement des radicaux libres par le diphényl-1,1 picrylhydrazyl-2 (DPPH). Les résultats montrent que les extraits d'éthanol de propolis obtenus au moyen de l'extraction par pression hydrostatique élevée et du lessivage à température ambiante ont des activités antioxydantes relativement fortes, ce qui peut être corrélé aux teneurs totales en polyphénol et flavonoïde. Les activités antioxydantes des extraits d'éthanol de propolis obtenues au moyen de l'extraction par pression hydrostatique élevée sont les mêmes que celles des extraits d'éthanol de propolis obtenus par le lessivage à température ambiante. Le lessivage à température ambiante nécessite généralement quelques jours, et peut même prendre plus de 7 jours, tandis que l'extraction par pression hydrostatique élevée ne nécessite que 1 min. Ces découvertes illustrent davantage que l'extraction par pression hydrostatique a un bel avenir pour l'extraction des flavonoïdes à partir du propolis.

Keywords: propolis, extraction methods, antioxidant activity

INTRODUCTION

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, has been used in folk medicines in many regions of the world and has been reported to have various biological activities such as antioxidant ability, antibacterial, antiviral, anti-inflammatory and anticancer properties (Kimoto et al., 2001). For this reason, propolis is extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart disease, diabetes and cancer (Banskota et al., 2001). Pharmacological activities, such as anticancer, anti-inflammatory, antibiotic, antioxidative, antiviral, antifungal, anaesthetic and cytostatic, have been ascribed to ethanolic extracts of propolis (EEP) (Nieva et al., 2000). Analysis of EEP showed the presence of antibacterial and free radical-scavenging activities (Nieva et al., 2000). EEP usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, and so on. The antioxidant activity of EEP may be related to polyphenol and flavonoid contents since it has been reported that these phenolic compounds can break the chain reaction of lipid, inhibit chemiluminescence reactions, scavenge several ROS (Krol et al., 1996), etc.

There are papers in literature that deal with the methods of extraction of flavonoids from propolis, such as leaching at room temperature (LRT) (Murad et al., 2002), and heat reflux extraction (HRE) (Gu et al., 2001). LRT is the most currently applied technique, but it usually needs a few days, and can take even more than 7 days. HRE generally needs a higher temperature (85°C) during the extracting, which can lead some heat-sensitive ingredients of propolis to lose their biological activity. High hydrostatic pressure extraction (HHPE) is a novel technique at present, which was successfully used in extracting flavonoids from propolis (Jun, 2005). Experiment results have

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shown HHPE has many advantages, such as shorter time (only 1 min), higher extraction yield and so on (Jun, 2005). Propolis obviously possesses antioxidant activity, as reported by many researchers. However, there are few studies on the comparison of the antioxidant activity of EEP obtained by HHPE, LRT and HRE. Therefore, the present study is aimed at the investigation of the comparison of the antioxidant activity of EEP obtained by different extraction methods in relationship to their total polyphenol and flavonoid contents.

MATERIALS AND METHODS

Materials and Instrumentation

Crude propolis that had been collected in Nongan County of Jilin Province (China), which was mainly from the Japan pagoda tree bud or unexpanded leaves visited by the bees, was provided by the Jilin Provincial Institute for Drug Control. Rutin and gallic acid, pharmaceutical grade standard, was purchased from the National Institute for Control of Pharmaceutical and Biological Products (China). Tween 80, ethanol, chloroform, aluminum chloride and potassium acetate (Beijing Chemical Reagents Company; analytical grade) were used. Tert-butylated hydroxyquinone (TBHQ), β-carotene, linoleic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich Co. (St. Louis, U.S.A.). The spectrophotometer (751-GW) was from Shanghai Analytical Instrument Overall Factory.

Ultrahigh pressure isostatic apparatus (DL700-0.55 × 1.5) was purchased from Shanghai Dalong Ultrahigh Pressure Machine Co., Ltd. (China) (Effective volume of vessel: 0.35 L, maximal working pressure: 700 MPa, inner diameter: 55 mm, pressure transmitting media: mixture of transformer oil and kerosene).

Preparation of EEP by HHPE

Crude propolis was frozen at −20°C and ground in a chilled disintegrator. Then, 10 g of crude propolis was mixed with 350 mL of 75% ethanol and placed into a sterile polyethylene bag. The bag was sealed after the air inside was eliminated. The bag was placed into a hydrostatic pressure vessel in an ultrahigh pressure isostatic apparatus. After being processed (high pressure levels: 500 MPa) for 1 min at room temperature, the mixture was filtered through filter paper. The extracts were centrifuged at 4000 × g for 10 min, and the supernatants were pooled. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were pooled. Supernatants obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40°C, and then the supernatant was lyophilized. Thus, the EEP by HHPE were prepared (Jun, 2005). A solution (10 μg/mL ethanol) was used as the sample solution for the following tests.

Preparation of EEP by LRT

The EEP by LRT was obtained as described by Murad et al. (2002). In brief, 10 g of propolis were suspended and extracted with 30 mL of 70% ethanol and shaken at room temperature for a week. The mixture was filtered through filter paper; extracts were centrifuged at 4000 × g for 10 min, and the supernatants were pooled. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were pooled. Supernatants obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40°C, and then the supernatant was lyophilized. Thus, the EEP by LRT were prepared. A solution (10 μg/mL ethanol) was used as the sample solution for the following tests.

Preparation of EEP by HRE

The EEP by HRE was obtained as described by Gu et al. (2001). In brief, propolis ethanol extracts were boiled (10 g of propolis, mixed with 40 mL of 95% ethanol in water) at boiling point, about 85°C, for 4 h (super boiling of the solution did not occur). The mixture was filtered through filter paper, the extracts were centrifuged at 4000 × g for 10 min, and the supernatants were pooled. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were pooled. Supernatants obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40°C, and then the supernatant was lyophilized. Thus, the EEP by HRE were prepared. A solution (10 μg/mL ethanol) was used as the sample solution for the following tests.

Total Polyphenol and Flavonoid Contents

Phenolic compounds are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity (Kahkonen et al., 1999). Propolis contains a wide variety of phenolic compounds, mainly flavonoids. Contents of flavonoid and other phenolic substance have been suggested to play a preventive role in the development of cancer and heart disease (Kahkonen et al., 1999). The Folin-Ciocalteau method and the $AlCl_3$ colouration are currently used to determine the total polyphenol and flavonoid contents, respectively (Liu et al., 2002; Luximon et al., 2002). In the present study, we applied these methods to determine the total polyphenol and flavonoid contents of EEP samples.

Total polyphenol content in EEP was determined by the Folin-Ciocalteau colourimetric method (Kumazawa et al., 2002). EEP solution (0.5 mL) was mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. EEP samples were evaluated at the final concentration of 10 μg/mL. Total polyphenol content was expressed as mg/g (gallic acid equivalents).

Total flavonoids content in EEP was determined by the method of Woisky and Salatino (1998). 0.5 mL of EEP solution, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 40 min at room temperature, the absorbance was measured at 415 nm. EEP samples were evaluated at the final concentration of 10 μg/mL. Total flavonoids content was calculated as rutin from a calibration curve.

β-Carotene Bleaching Method

This experiment was carried out by the method of Emmons et al. (1999). β-Carotene (3 mg) was dissolved in 30 mL of chloroform, and 3 mL were added to 40 mg of linoleic acid and 400 mg of Tween 80. Chloroform was removed under a stream of nitrogen gas. Distilled water (100 mL) was added and mixed well. Aliquots (3 mL) of the β-carotene/linoleic acid emulsion were mixed with 50 μl of EEP solution and incubated in a water bath at 50 μl. Oxidation of the emulsion was monitored spectrometrically by measuring absorbance at 470 nm over a 60 min period. The control sample contained 50 μl of solvent in place of the extract. The antioxidant activity is expressed as percent inhibition relative to the control after a 60 min incubation using the following equation:

$$AA = 100\ (DR_C\text{-}DR_S)/DR_C \quad (1)$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control ($=\ln(a/b)/60$), DR_S is the degradation rate in the presence of the sample ($=\ln(a/b)/60$), a is the initial absorbance at time 0, and b is the absorbance at 60 min. EEP samples were evaluated at the final concentration of 10 μg/mL.

Free Radical Scavenging Activity on DPPH

The scavenging activity of the EEP on DPPH radicals was measured according to the method of Chu et al. (2000) with some modifications. An aliquot of 2 mL of 1×10^{-4}mol/L DPPH radical in ethanol was added to a test tube with 2 mL EEP sample solution (10 μg/mL ethanol). Ethanol was used instead of the EEP sample solution as a control. The reaction mixture was incubated for 1 h at room temperature and the absorbance (Abs) was determined immediately after mixing by measuring at 517 nm with a spectrophotometer. The scavenging activity (%) (SA) on DPPH radicals was calculated by Equation (2):

$$SA = 100\ (1\text{-Abs in the presence of sample/Abs in the absence of sample}) \quad (2)$$

RESULTS AND DISCUSSION

Total Polyphenol and Flavonoids Contents of EEP

Propolis is commercially available as tinctures or tablets made from ethanol extracts in many countries. The total polyphenol and flavonoid contents are reported to be the most abundant and most effective antioxidant in propolis (Scheller et al., 1990). Therefore, we firstly investigated the total polyphenol and flavonoid contents of EEP obtained by different extraction methods.

Table 1 shows the total polyphenol and flavonoid contents of different EEP samples. The EEP by HHPE and LRT had no significant difference ($P < 0.05$), and the EEP by HRE showed the lowest value for polyphenol and flavonoid contents. Impurities, such as beeswax, which could not or had not been fully dissolved in ethanol solution at room temperature, were largely dissolved at high temperature (Cao and Wei, 2002). Thus, the impurity content of EEP would increase, which would lead to a decrease of total polyphenol and flavonoid contents. Therefore, the total polyphenol and flavonoids contents of EEP obtained by HRE were low.

Effects of Various EEP Samples on β-Carotene Bleaching Method

Figure 1 shows the antioxidant activity of EEP samples obtained by different extraction methods determined by β-carotene bleaching method. The antioxidant assay, using the discolouration of β-carotene is widely used, because β-carotene is extremely susceptible to free radical-mediated oxidation. β-carotene is discoloured easily by the oxidation of linoleic acid, due to its double bonds being sensitive to oxidation (Singh et al., 2002). EEP samples were evaluated at the final concentration of 10 μg/mL for the assay.

As shown in Figure 1, EEP by three extraction methods had strong antioxidant activity—over 60%. EEP samples obtained by HHPE and LRT had stronger antioxidant activity than that by HRE. The EEP by HRE, which presented lower total polyphenol and flavonoid contents, exhibited weaker antioxidant activity.

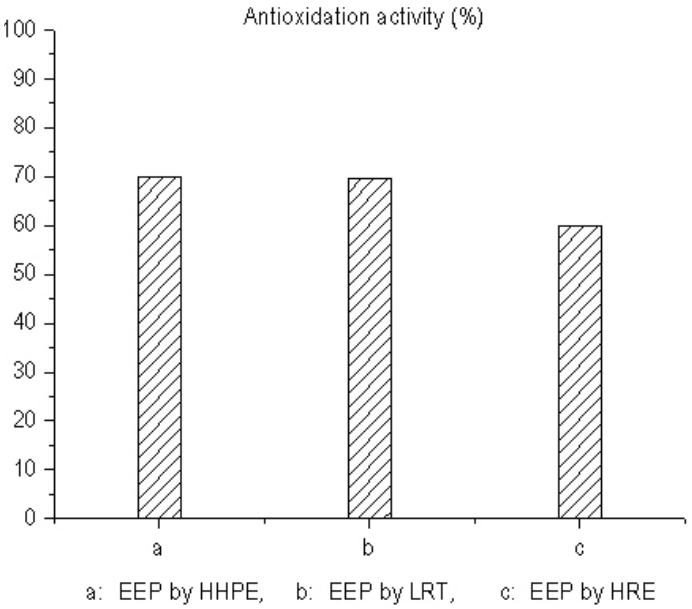

Figure. 1. Antioxidant activity of EEP (a–c) obtained by different extraction methods in the β-carotene-linoleic acid system. Values are means ± standard deviations of triplicate measurement ($P<0.05$, Student's t-test).

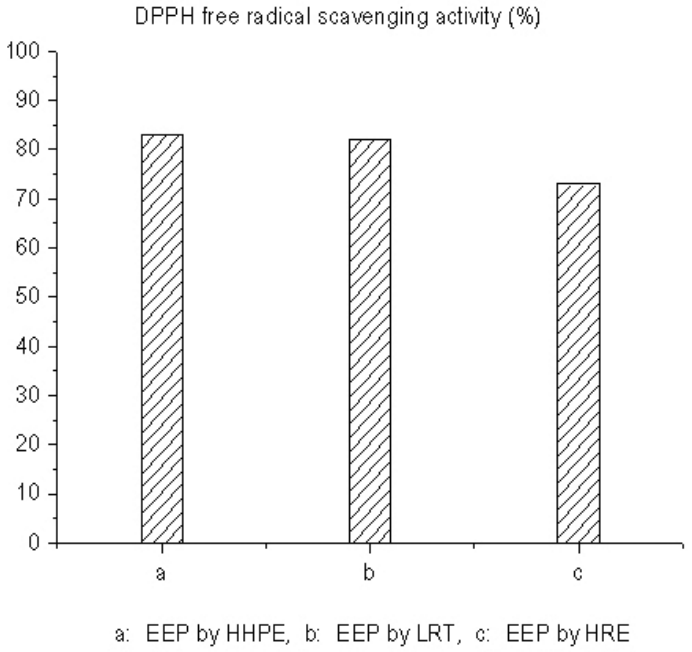

Figure. 2. DPPH radical scavenging activity of EEP (a–c) obtained by different extraction methods. Values are means ± standard deviations of triplicate measurement ($P<0.05$, Student's t-test).

The antioxidant activity shown in Figure 1 seemed to correlate with the total polyphenol and flavonoid of EEP (Table 1). Positive correlations were found between total polyphenol and flavonoid contents in the EEP and their antioxidant activities. Phenolic compounds, such as flavonoids, are the type of antioxidant that possesses a strong inhibition effect against lipid oxidation through radical scavenging. Flavonoids have been reported to be the most abundant and most effective antioxidant in propolis (Isla, 2001). There are many papers concerning the

antioxidant activity of the ethanol extract of propolis, which has been attributed to the high content of flavonoids in propolis (Pascual et al., 1994; Chen and Ho, 1995).

Effect of Various EEP Samples on DPPH Free Radical

The DPPH free radical scavenging activity of various EEP samples is shown in Figure 2. The model system of scavenging DPPH free radical is a simple method for evaluating the antioxidant activity of compounds. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability (Tang et al., 2002). We evaluated various EEP samples at the final concentration of 10 μg/mL.

As shown in Figure 2, EEP samples by three extraction methods had strong DPPH free radical scavenging activities of over 60%. EEP samples by HHPE and LRT had stronger DPPH free radical scavenging activities than that by HRE. These EEP samples had high total polyphenol and flavonoid contents (Table 1). EEP sample by HRE, which had weaker antioxidant activities in the assay system using the discolouration of β-carotene (Figure 1), exhibited weaker DPPH free radical scavenging activity. We also found that the DPPH free radical scavenging activity shown in Figure 2 seemed to correlate with the antioxidant activity shown in Figure 1. The EEP sample with strong antioxidant activity also has strong DPPH free radical scavenging activity.

Table 1. Total polyphenol and flavonoid contents of EEP obtained by different extraction methods

Extraction methods	Flavonoids content of EEP (mg/g)	Total polyphenol content of EEP (mg/g)
HHPE	230.4±6.5 a	290.4±8.7 a
LRT	232.1±3.2 a	296.0±6.4 a
HRE	167.9±5.5 b	247.7±7.0 b

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means with different letters (a, b) were significantly different ($P < 0.05$, Student's t-test).

CONCLUSIONS

In this study, the antioxidant activity of various EEP samples obtained by three extraction methods (HHPE, LRT and HRE) was investigated. The results showed that the EEP obtained by HHPE and LRT had relatively strong antioxidant activities, which may be correlated with the high total polyphenol and flavonoid contents. Antioxidant activities of EEP obtained by HHPE were the same as those of EEP obtained by LRT. LRT usually needs a few days or can take even more than 7 d, while HHPE needs only 1 min. These findings further illustrate that HHPE has a bright prospect for extracting flavonoids from propolis.

HHPE is suitable for the extraction of flavonoids from propolis, not only because it is more rapid, safer and eco-friendly than conventional extraction methods, but also because its extract has strong antioxidant activity. Food and medicinal industries will benefit from this emerging technology.

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